THE CYTOCHEMICAL STAINING AND MEASUREMENT OF PROTEIN WITH MERCURIC BROMPHENOL BLUE [1]

DANIEL MAZIA, PHILIP A. BREWER AND MAX ALFERT

Department of Zoology, University of California, Berkeley 4, California

Various techniques have been adapted from protein chemistry to the cytological demonstration of proteins (*e.g.*, ninhydrin reaction, Mazia and Jaeger, 1939; Millon reaction, Pollister and Ris, 1947; Sakaguchi reaction, Thomas, 1946; Serra, 1946) but none has found wide use for both the resolution of morphological detail in terms of protein distribution and the measurement of relative protein concentrations. Danielli (1948) has presented an incisive summary of the problem and has proposed some ingenious new approaches, but these have not yet found common application.

The development of chromatographic and electrophoretic techniques which require identification of spots on filter paper has given new impetus to the study of color reactions of amino acids and proteins, and it is inevitable that the experience which is developing rapidly in this field will be carried over to the field of biological staining. Durrum (1950) devised the mercuric bromphenol blue reagent which has been widely adopted for the detection of protein spots, and the reaction has been studied in some detail by Kunkel and Tiselius (1951) and Geschwind and Li (1952). The present study deals with the application of the mercuric bromphenol blue procedure to cytological material. Its value as a simple staining technique, its capacity for bringing out in good contrast certain structures that often do not stain well by other procedures, and its specificity and applicability to the photometric estimation of proteins in cytological preparations will be considered.

Experimental

1. *Preparation of tissue.* Common cytological fixatives, such as Carnoy's, Baker's formalin, Schaudinn's and Bouin's solutions, have been employed in this study. Of all fixatives tested, only those containing osmium interfere with this reaction. The alcoholic mercury-bromphenol blue reagent (Hg-BPB) as described by Durrum would in itself have the properties of a fixative so that theoretically fixation and staining could be carried out in one step. We have found in practice that fixation of blocks of tissue proceeds too slowly in this solution, and the slow penetration sets up artifacts in protein distribution due to the transport of unprecipitated protein within the cells by the flowing fixative. However, we have found the Hg-BPB solution to be satisfactory in the fixation of cell smears and of cilia (Fig. 1). Presumably it would be adapted to other surface structures.

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It is necessary to work with quite thin preparations because the staining by this method is so intense in most cases. Sections of the order of 5 microns or less are recommended.

2. *Staining.* Staining may be carried out either in the alcoholic Hg-BPB solution described by Durrum or the aqueous solution used by Kunkel and Tiselius. In both cases the solution we have used contains 10 grams of $HgCl_2$ and 100 milligrams of bromphenol blue per 100 ml. The alcoholic solution is made up in 95 per cent ethanol. The preparations are immersed in the dye solution for 15 minutes.

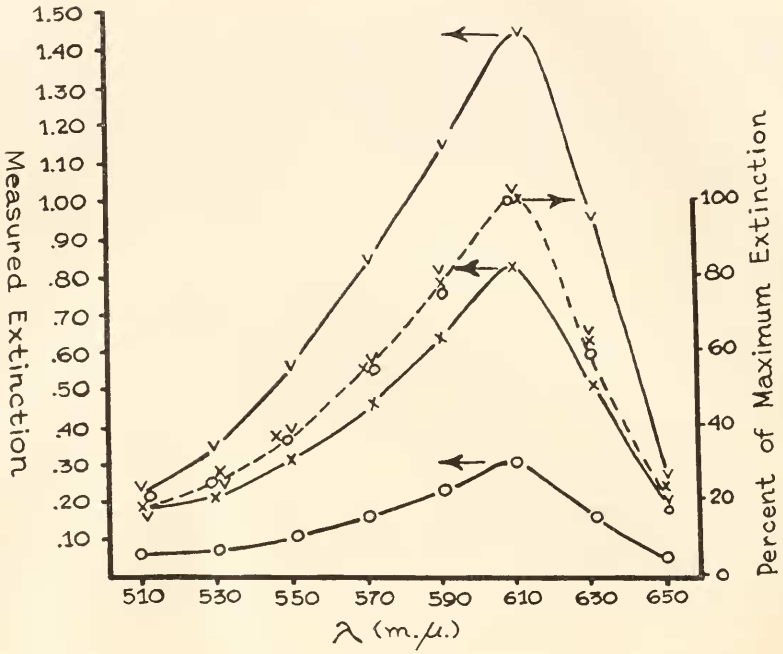


FIGURE 1. The validity of Beer's Law for the comparison of the Hg-BPB staining of objects of different optical density. Complete absorption spectra are given for the nucleolus of a sea urchin (*Strongylocentrotus purpuratus*) oocyte (—v—), cytoplasm of a rat intestinal mucosa cell (—x—) and cytoplasm of a mature sea urchin egg (—o—). The data on the egg cytoplasm are not comparable to Tables I and II since a thinner region was chosen in order to obtain a curve in a lower extinction range. In all three cases shown, a region 3 microns in diameter, producing a 3 mm. spot at the phototube level, was measured. Slit width: 2 mm.

A longer period does not increase the intensity of staining. The preparations are then washed for 20 minutes in 0.5 per cent acetic acid as described by Kunkel and Tiselius. This solution removes excess dye but does not remove the dye that is presumably bound chemically, even after 5 hours of washing. Washing in water for 15 minutes is perfectly suitable for cytological purposes, but there is no sharp end point; with prolonged treatment with water more and more dye is removed. After the acid wash, the sections are immersed in water or buffer of pH 6–7 for three minutes to convert the dye to its blue alkaline form. Treatment with ammonia

vapor, as recommended for filter paper strips, is not suitable as it produces serious distortion of morphological detail.

These three steps constitute the whole procedure and yield reproducible results. All other standard manipulations appropriate to making temporary or permanent mounts may be carried out without apparent effects on the staining of the cells, except that prolonged soaking in dilute ethanol during dehydration may lead to some loss of dye.

Results

1. *Qualitative.* A variety of materials, plant and animal, has been studied. The procedure described is believed to be of general applicability as a cytological and histological technique. In the material we have studied, there was no known structure that was not brought out in good contrast to its background by the technique, and this is not at all surprising since it is difficult to imagine the existence of a cell structure without some concomitant differentiation in protein composition and concentration. Each of the structures selected for purposes of illustration may, of course, be stained by other means, but for the most part different techniques would be required in different cases. Fixatives and stain solutions employed are designated in the legends to the photographs.

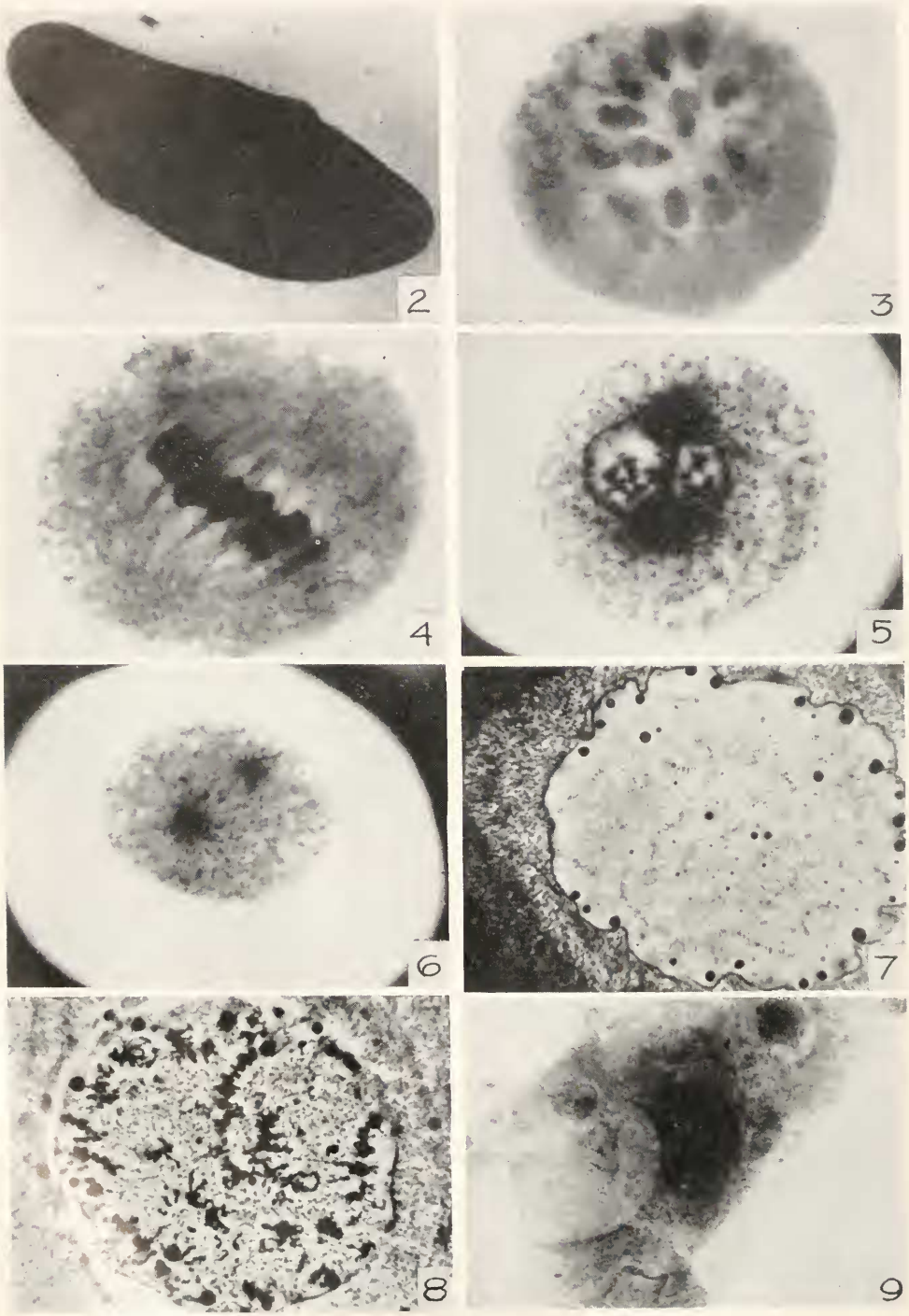
(1) *Cilia.* Figure 2 shows a *Paramecium* fixed and stained directly in the alcoholic Hg-BPB solution. This cell is too thick for the observation of internal detail but the cilia, which ordinarily require special staining techniques, appear dark and distinct.

(2) *Mitotic figures.* Figures 3 and 4 show microspore divisions in *Lilium longiflorum.* Figure 2 is a metaphase plate and is of special interest because the regions of attachment to the spindle fibers seem to be distinguished from the other regions of the chromosomes by their greater dye-binding capacity. Figure 3 is a later stage in equatorial view, showing strong staining and apparent structural detail of the spindle components. Each chromosome seems to be connected to the polar cytoplasm by a bundle of fibrils.

Figure 5 shows an early stage in the first division of an egg of *Anascaris equorum.* The material of the "achromatic figure" (by this technique the most densely staining structure in the cell) is seen in the form of two asters connected by a mass of staining material lying between the adjacent pronuclei. In Figure 6, an equatorial view of an early anaphase in the first cleavage of the same form, the complete mitotic figure, with asters, spindle and two chromosomes, is seen.

Dr. Sajiro Makino (personal communication) has applied this technique to the demonstration of meiotic figures in orthopteran and hemipteran testes with satisfactory results.

(3) *Lamp brush chromosomes.* The giant "lamp brush" chromosomes found in the oocytes of amphibian and other lower vertebrates are difficult to stain by most methods because the nucleic acid is rather dispersed. The Hg-BPB technique brings out their structure clearly. Figure 7 shows an oocyte of the frog *Rana pipiens* and Figure 8 an oocyte of the urodele *Batrachoceps attenuatus.* In the latter case, the chromosomes are more condensed and therefore more conspicuous. The frog chromosomes are highly extended, but the stain differentiates their characteristic organization as a thread with long lateral projections or loops rather well.



FIGURES 2–9.

This picture is interesting because it corresponds so well to that obtained, in more favorable species, in living nuclei (Duryee, 1950).

(4) *Amoeba.* In our experience, the nucleus of *Amoeba proteus* is brought out only in very poor contrast with nucleic acid stains. The concentration of nucleic acids seems to be relatively low in this large nucleus. Figure 9 shows an individual *Amoeba proteus* stained by the Hg-BPB technique, showing the nucleus to be a structure where the protein concentration is much higher than that obtaining in the cytoplasm.

These preparations, as well as others we have obtained, contain a number of features of considerable cytological interest, which will not be discussed at this time. Some will be evident from inspection of the figures. The crispness and clarity of the results have been noteworthy if only because they are obtained by a rapid method that requires no modification from one type of material to another.

2. *Quantitative.* Preliminary to a study of the specificity of the Hg-BPB stain, a series of measurements has been made to determine whether the Beer and Lambert laws apply to material stained by this method. The apparatus and technique were those previously described by Alfert (1950), except that a Beckmann Model B spectrophotometer was used as a light source.

The absorption maximum of the dye-protein complex in tissue is 610 mμ. The test of the applicability of Beer's Law was carried out in two ways. First, extinction measurements at various wave-lengths were made on three different regions within the same cell: the cytoplasm, nucleus and nucleolus of oocytes of the sea urchin *Strongylocentrotus purpuratus.* In Table I the absolute and relative extinction values are given for four wave-lengths. The measurements given in the table were made on the same cell, cut at 5 microns. Therefore the extinction values for different regions are comparable. The "relative" values were calculated by letting the measured extinctions at 610 mμ equal 100. It is seen that while the intensity of staining varies from one region to another, the relative extinctions are nearly the same at different wave-lengths, as required if Beer's Law is valid for the situation. Figure 1 leads to the same conclusion by a somewhat different approach. Complete absorption curves were taken for three materials—

FIGURES 2–9.

FIGURE 2. *Paramecium caudatum.* Fixed and stained in aqueous Hg-BPB. Whole mount. × 330.

FIGURE 3. *Lilium longiflorum.* Microspore division. Polar view of metaphase plate. Fixation: Carnoy's solution. Sectioned at 8 μ. Stain: aqueous Hg-BPB. × 1240.

FIGURE 4. *Lilium longiflorum.* Microspore division. Equatorial view of anaphase. Fixation, sectioning, and staining as in Figure 3. × 1330.

FIGURE 5. Egg of *Anascaris equorum.* Stage of close approach of pronuclei. Fixation: Carnoy's solution. Section at 8 μ. Stain: aqueous Hg-BPB. × 970.

FIGURE 6. Egg of *Anascaris equorum.* Equatorial view, first cleavage division. Note staining of asters, spindle, and the two chromosomes. Prepared as in Figure 5. × 830.

FIGURE 7. Ovary of *Rana pipiens.* "Germinal vesicle" of oocyte. Note staining of nucleoli and fine "lamp brush" chromosomes. Fixation: Bouin's solution. Sectioned at 10 μ. Stain: aqueous Hg-BPB. × 300.

FIGURE 8. Ovary of *Batrachoceps attenuatus.* "Germinal vesicle." "Lamp brush" chromosomes seen in longitudinal and transverse section. Note structure of projecting loops. Fixation: Bouin's solution. Sectioned at 10 μ. × 350.

FIGURE 9. Part of an *Amoeba proteus,* showing intense protein-staining of nucleus. Fixation: Carnoy's solution. Squash preparation. Stain: aqueous Hg-BPB. × 430.

TABLE I

The validity of Beer's Law for the Hg-BPB staining of cell contents (sea urchin oocyte)

Wave-length	Extinction					
	Cytoplasm		Nucleus ("sap")		Nucleolus	
	Measured	Relative	Measured	Relative	Measured	Relative
530	0.187	33	0.194	36	0.265	27
570	0.314	55	0.314	59	0.606	61
610	0.568	100	0.537	100	1.00	100
650	0.068	12	0.089	17	0.052	15

the sea urchin oocyte nucleolus, the cytoplasm of mucosa cells of rat intestine, and the cytoplasm of the mature sea urchin egg. The extinction values at 610 mμ range from 0.3 to 1.44. The absolute extinction curves are evidently parallel, but the close parallelism is best shown in curve 4, where the relative extinctions for all three materials are plotted on the same scale by setting the maximum value as 100. Over such a wide range of extinction values, deviations from Beer's Law through recognized mechanisms (Sandell, 1944) would be expressed as changes in the absorption curves.

A rather elegant means of testing the applicability of Lambert's Law was provided by sectioned sea urchin egg populations, where we frequently encountered cases in which sections of two cells slipped and partially overlapped. It was thus possible to compare the absorption by single sections of two eggs with the absorption of the regions where these were superimposed. The results are given in Table II where it is seen that the extinction values are additive to a satisfactory degree. The several tests listed in Table II were made on different pairs of cell-sections. Regions 4 microns in diameter were measured, giving a 4 mm. spot at the level of the phototube.

In visual inspections of slides stained by this method, it often appeared that the most intensely stained preparations also seemed to have a more reddish hue. At

TABLE II

Tests of the validity of Lambert's Law for measurement of Hg-BPB staining of sea urchin egg cytoplasm. Measurements made on adjacent 5 micron sections (a and b) and on region where they overlap (a + b)

Test No.	Extinction at 610 mμ			
	Section a	Section b	Measured a + b	Deviation from calculated a + b
1	0.602	0.613	1.202	−1%
2	0.612	0.777	1.284	−8%
3	0.614	0.665	1.362	+6.5%
4	0.754	0.599	1.237	−9%
5	0.612	0.615	1.236	+1%

first this seemed to suggest the complication of metachromasia or some related effect. However, the photometric measurements revealed no difference between the bluer and the redder preparations. The reddish appearance of densely stained material may be explained in terms of the spectral sensitivity of the human eye, but is irrelevant to the present discussion since it has no influence on objective measurements.

The demonstration of the validity of photometric laws does not in itself imply that we are measuring the concentration of any normal constituents of the cell. In discussing this common misconception in relation to Beer's Law, Sandell (1944, p. 61) says, ". . . this law simply states that the extinction must be proportional to the concentration of the colored substance; it does not state that the extinction must be proportional to the analytical concentration of the constituent which forms the colored substance." Such an assurance can only come from chemical investigation of the staining reaction, as described below. But the above data do indicate that, insofar as we can interpret the dye-binding reaction, the microphotometric measurements will accurately follow its stoichiometry.

Since the Millon reaction has proved to be useful in photometric studies on cell proteins, it is of interest to compare it with the Hg-BPB reaction. A disadvantage of the Millon technique is that the compound formed absorbs so weakly in the visible. While satisfactory extinctions may be obtained in the near-ultraviolet

TABLE III

The binding of Hg-BPB by various substances on filter paper

Substance	Moles dye bound per mg.
Starch	0
Glycogen	0
DNA	5.9×10^{-9}
Cholesterol	5.4×10^{-9}
Bovine serum albumin (cryst.)	1.20×10^{-6}
Chymotrypsin (cryst.)	1.06×10^{-6}
Pepsin (cryst.)	1.03×10^{-6}
Histone (pH 4)	1.40×10^{-6}

region, the structures to be measured are difficult to locate visually. We have compared the extinction values of sea urchin egg cytoplasm stained by the Millon procedure (measured at 355 mμ) and by the BPB procedure (measured at 610 mμ). The average of 10 measurements by the BPB procedure for a given series of sections was 0.646. The Millon reaction gave an average extinction of 0.121. Thus the BPB reaction provides greater optical density with a given concentration of material as well as an absorption maximum in a more convenient range.

3. *Specificity and quantitative significance.* Few tests for protein actually measure the mass of protein. What is usually determined is some chemical group that is characteristic of proteins. The tests give information concerning the amount of protein present only insofar as one has independent information as to the distribution of the particular group or groups in the protein under observation.

Obviously, the first consideration is the qualitative specificity of the BPB reaction; whether the dye is combined by non-protein constituents of the cell. We have tested, on filter paper, the dye-binding by a series of substances that might confuse the reaction. The following table (Table III) indicates that the dye-

binding by the non-protein materials tested is negligible compared with that of protein. The procedure was exactly as described above, carried out on filter-paper spots. The amount of dye was estimated after extraction of the paper with ammoniacal acetone, the standard being a known solution of dye in the same solvent.

The question of the BPB reaction of amino acids and polypeptides has been studied in detail by Geschwind and Li (1952). From their work it appears that many free amino acids and peptides would bind dye, but the dye complexes—with the exception of those involving histidine and possibly cysteine—would be dissolved away during the washing of the preparation.

From the cytological standpoint, one is most interested in the possibility of distinguishing protein in structures containing nucleic acid. We therefore studied the BPB reaction in cells from which nucleic acids had been removed by extraction with hot 5 per cent trichloroacetic acid for 15 minutes. No decrease in the BPB staining is observed. In fact, certain structures, especially the chromosomes, now stained more intensely, as would be anticipated if the nucleic acid were blocking potential dye-binding groups. The fact that the intensity of chromosome staining increased is consistent with the view that many of the basic groups of nuclear proteins are bound to the acid groups of nucleic acid.

Kunkel and Tiselius (1951) have shown that the amount of dye bound is proportional to the amount of protein over a wide range. They found, however, a 30 per cent difference in the dye-binding per milligram of albumin and gamma globulin. We have tested a number of pure proteins and mixtures containing proteins (tissue homogenates) on filter paper and have consistently observed the same linearity and variability. Our highest and lowest values for dye bound per milligram protein differ by about 40 per cent. Our procedure differed from that of Kunkel and Tiselius only in that the dye was extracted from the paper by ammoniacal acetone. On the average, one milligram of protein combined about 10^{-6} moles of dye. Taking 120 as a representative weight of an amino acid residue, this would mean that one molecule of dye is bound for, approximately, every 10 amino acid residues present.

In view of this variability the method will be applicable to the exact comparison of total protein concentrations only where the qualitative differences are expected to be small. It will be suitable for the approximation of large differences, since the variation of dye-binding among various proteins studied is by no means one of order of magnitude.

The variability is true of most protein methods, which generally involve the measurement of certain characteristic groups in proteins. It does not seem likely, from the results reported, that the method is specific for a single group. As mentioned, we have found that in a variety of proteins the ratio of moles of dye bound to amino acid residues present is roughly 0.1. Examining the composition of the proteins, we find no single type of amino acid that accounts for 10 per cent of those present.

If we examine those groups which could potentially enter the reaction, these seem to be the NH_2 groups of basic proteins, which might combine by direct salt formation, and the SH groups, aromatic groups and free COOH group which might be coupled to the dye through the mercury.

The experiments showing enhanced staining of chromosomes after extractions

with trichloroacetic acid indicate that the basic groups of basic proteins may contribute to the dye-binding. This would be a simple case of acid-staining, in the microscopists' terminology, and would not be expected to involve combination with mercury. A number of experiments, both with pure proteins on filter paper and with tissues, suggests that this direct combination of the dye with basic groups is, in fact, largely limited to basic proteins, but that a mechanism involving coupling through Hg is responsible for the major part of the staining of tissues and of ordinary proteins. In the work with pure proteins on filter paper it was shown that spots of protamine stained equally well whether the Hg was present or absent. On the other hand, ovalbumin stained only in the presence of Hg; when Hg was omitted, there was a loose attachment of dye which readily washed out in the standard procedure. Since the function of the Hg might merely be that of keeping the protein on the paper, we returned the albumin-containing papers, which had been washed free of dye after treatment with the Hg-free reagent, to the normal Hg-containing dye solution. The spots now stained normally and the dye could not be washed out. Therefore the Hg plays a role in coupling the dye to the protein. In these experiments, the protein spots were fixed with 3 : 1 alcohol-acetic acid and dehydrated with alcohol.

Exactly the same relationship held for tissues. When these were treated with a dye solution without Hg, they seemed to take up a considerable amount of dye, but most of it was removed on washing. Under the microscope, a typical picture of acid-staining was observed. It is suggested that a comparison of staining with and without Hg, and before and after the extraction of nucleic acids, might provide a basis for estimating the relative amount of basic protein and the extent to which the basic groups were free, but this has not been investigated in detail.

The groups which might combine the dye through Hg, the SH, aromatic and free COOH groups, might be differentiated by blocking with appropriate group-reagents, and it is proposed to investigate this possibility.

Discussion

For the cytochemist who is interested in estimating the total concentration of protein, rather than some characteristic side chain, the ideal method would be one in which each amino acid or peptide linkage would produce an equivalent light absorption. Such a method would be provided by the biuret reaction, for instance, though the extreme alkaline conditions required render this unsuitable for cytological purposes. The next best approximation is a method which will register such a variety of groups as to minimize, by a statistical "averaging-out," the consequences of compositional differences among proteins. The Hg-BPB method would seem to have this advantage. Inspection of tables of protein composition shows that the aggregate percentage of the amino acids potentially reacting in this method varies less from protein to protein than the percentage of any one amino acid.

The method has the further advantages that there is no class of proteins that will not be stained significantly and that, insofar as the composition of the test-object is known, the quantitative differences in the dye-binding by different proteins may be assessed accurately by parallel studies on filter paper. The studies that have already been made by Kunkel and Tiselius and by ourselves show that proteins and protein mixtures may readily be analyzed by simple filter-paper techniques with

excellent correlation between the amount of protein present and the amount of dye bound. The variation that we have discussed previously seems to be a genuine index of qualitative differences among proteins, and subject to analysis.

The fact that the dye is combined to different groups and by at least two different mechanisms offers certain interesting possibilities. For instance, the staining without Hg appears to be a typical case of acid staining, which has been used by others (*e.g.*, Schrader and Leuchtenberger, 1950) as a measure of basic proteins. If bromphenol blue is used, and the staining in the presence and absence of Hg is compared, one has a system of comparison of basic protein to total protein in which at least the photometric units (extinction, optical density) can be compared directly in terms of the number of dye-binding groups. This would not be the case if different dyes were used for the two classes of proteins. When it becomes possible to sort out the various groups that combine the dye through Hg, the same advantage will obtain. In this way, the Hg-BPB method should be a useful complement to those methods which are selective for one group, such as the Millon method for phenolic residues and the methods specific for SH groups (Tahmisian and Brues, 1951; Bennett, 1951).

Summary

1. The mercuric bromphenol blue reaction as used for development of protein spots on filter paper has been found to be applicable to the cytological staining of proteins.

2. The optimum procedure is identical in detail with that described by Kunkel and Tiselius for filter paper spots, except that a neutral aqueous solution is substituteed for ammonia vapor in the final color development.

3. The sharp and intense staining of protein permits good differentiation of structures often difficult to observe, such as cilia, spindle elements, regions of spindle fiber attachment to chromosomes and "lamp brush" chromosomes.

4. The procedure is specific for proteins and those peptides which are not removed in the washing procedure.

5. The preparations stained by this procedure follow the Beer and Lambert Laws in microspectrophotometric measurements. The absorption maximum is at 610 millimicrons.

6. Basic proteins bind the dye under the conditions of the method even when Hg is omitted. Other proteins bind the dye by coupling through Hg. As expected, structures containing basic proteins show enhanced staining after removal of the nucleic acid.

7. The number of groups in various proteins binding the dye in this procedure varies somewhat, but the average is about one dye-binding group per 10 amino acid residues.

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